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CZECHOSLOVAK STUDIES ON DIAGNOSIS OF BRUCELLOSIS

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FOREWORD

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CZECHOSLOVAK STUDIES ON DIAGNOSIS OF BRUCELLOSIS

[Following are translations on the above subject, selected from a Czechoslovak source. Source information accompanies each article.]

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BRUCELLOSIS GRANULOMAS IN SHEEP AND LABORATORY
ANIMALS IN RELATION TO DIAGNOSIS

Following is the translation of an article by Jozef Bogdan
in Veterinarni medicina (Veterinary Medicine) issue No 11,
Prague, November 1960, pages 863-868.

Introduction

The diagnosis of sheep brucellosis is often rather difficult, both in animals and in men. Serological and allergic tests are often unsuccessful. In cases of suspicious symptoms and changes, an isolation of the germs is necessary for the final diagnosis. A large majority of authors noted phenomena characteristic of sheep brucellosis in the organs of the reticulo-endothelia system (RES); these changes have been the subject of our investigation both in sheep and in laboratory animals as far as the formation of granulomas is concerned.

Bibliography

There are not very many records on any specialized research in the granulomatose infections of sheep. Cernjak and Juskovec noted them in sheep lungs, liver, cerebral ganglia, and testes. They were also noted by Ariel, but he does not consider these granulomatose infections specific.

Other authors who noted brucellosis granulomas in some other domestic and laboratory animals were Brown, Forbus, and Kerby; Christiansen and Thomsen; Dmitrijev; Andrejev; and Bol', who described them in swine spleen, liver, and cerebral ganglia.

Bol' noted them in bovine RES organs, uterus, and lungs; Bendtsen, Christiansen, and Thomsen; Roux and Bouvier; and Tudoriu, in the rabbit spleen, liver, uterus, lungs, and testes. The most frequent occurrence of granulomas is noted in guinea pigs, in their RES organs as well as other organs. They were described by Versilova and Kokorin; Nikolajev; Juskovec; Kolesnikov; Huddleson and Fabyan; Lofler, Moroni, and Frey; Harris; Braude; Roulet; Boyd; and Moulton and Meyer.

Ljubasenko noted them in foxes; Brown, Forbus, and Kerby, in dogs; Juskovec, in poultry.

Some authors, e.g. Lofler, Moroni, and Frey; Christiansen and

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Thomsen; Roulet; Kohler; and others place them into the category of specific granulomas.

A number of reports on the morphology of the brucellosis granulomas can be found in human [non-veterinary] literature; here Lofler, Albertini, and Lieberherr; Cortese; Vegener; Aiello; Steiger; Abrikosov and Strukov; Harris; Bell; and Willis may be mentioned.

A summary of the data concerning the structure of the brucellosis granulomas is given in Table I. It is evident from this table that according to the occurrence of the final elements of brucellosis granulomas, the most stable elements of the mentioned granulomas are epithelioid and giant cells of various types. Even other cell elements, especially eosinophil granulocytes, are a relatively significant source.

Material and method.

In the investigation of the brucellosis granulomas, 304 animals, including sheep as well as laboratory animals (especially guinea pigs, rabbits, white rats, and white mice), were used. Subjected to tests were 35 sheep, 31 rams, 24 rabbits, 74 guinea pigs, 20 rats, and 20 white mice. The above-mentioned kinds of animals were experimentally infected by species *B. suis* and *B. abortus*, and also by individual brucellosis species which had been isolated from infectious epididymis of rams by Nixnansky. In each group, control animals were used. The experimental contamination was done parenterally. The intraperitoneal contamination in guinea pigs and rats was done with a dose of 0.5 ml [milliliter], in rabbits with 0.6 ml, in white mice with 0.2 ml in a bacterial suspension stopped colorimetrically at a certain density through the application of Brown's barium-sulfate solution. Sheep and rams were experimentally infected in various ways with the individual brucellosis species with 2.5 ml doses. In the course of the investigation and before the animals were killed, serological and allergic tests for brucellosis were given. After the killing or after the animals died themselves, bacteriological testing was done by the SVU (Štátny výzkumný veterinárny ústav -- State Veterinary Research Institute) of Bratislava and its branch in Kosice.

After death, dissection and coprological examination for the presence of parasites were done; samples for bacteriological examination and the excised, changed parts for histological examination were taken. Fixation was done in 10% formaldehyde, Zenker's fluid and alcohol. Current coloring by hematoxyline-eosine and, according to the need, additional coloring methods by the usual histochemical processes as well as Lofler's methylene blue indicative bacterial coloring method, Nicole's carbolfuchsin, and Gram's method (in Weigert's adaptation) were used.

Results

The morphological examination of various organs in which granulomas and other morphological changes in subjects which fell ill spontaneously or were experimentally infected, occurred as shown in table 2. It demonstrates that both in the sheep and laboratory animals, granulomas were

Table 1

The structure of brucellosis granulomas according to the results of the authors' investigation.

Author	Occur- rence	Organ	Granuloma Structure															Notes
			Lymphoid	Epitheloid	Plant cells	Neutrophil poly-	Eosinophil	Lymphocytes	Histiocytes	Plant stray cells	Reticular	Plasmatic	Monocytes	Vessels	Erythrocytes	Collagen fibrils	Other types of cells	
Abrikosov, Strukov A. I. 1953				+	+	+		+									Dispersed epitheloid cells	
Juskovec M. K. 1952	0	Lungs	+	+													Necrosis in the center	
Juskovec M. K. 1952	0	Liver	+	+													In the center necrosis is surrounded by cover of polyblasts and reticular fibrils	
Juskovec M. K. 1952	0	Lymph. gangl.		+													The center necrotizes	
Juskovec M. K. 1952	0	Seminal vesicle		+													Non-specific granuloma	
Ariel M. M. 1939	0																The center necrotizes; Langhans giant cells and stray cells	
Brown, Forbes, Kerby 1945	5				+													

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Brown, Forbus, Kerby 1945	S	Spleen																	Unidentified center does not show cal- cification. Langhans giant cells and stray cells
Brown, Forbus, Kerby 1945	S	Lymph. gangl., spleen, liver	+	+															Necrosis in the center
Christiansen N., Thomsen A., 1956																			Necrotic center is surrounded by gran- ular tissue contain- ing giant cells
Dmitrijev A. I. 1948	S	Liver, lymph. gangl., suprarenal glands, lungs, spleen, uterus.																	
Andrejev																			Intrapulpose gran- uloma
Forbus	S	Cerebral gangl., kidneys, marrow																	Granulomas imper- ceptible by sight.
Bel K. G. 1948	S	Uterus	+	+															The center necro- tizes
Bel K. G. 1954	B	RES, uterus, lungs	+	+															
Roux, Bouvier A., 1948	Cu																		The center necro- tizes

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found mainly in the RES organs, but also in the reproductive system.

In 35 sheep infected in various ways and with various species, lymphoid granulomas were found in lungs (12) liver, (19) eosinophil granulomas in liver (16), lungs (1); epitheloid granulomas in lungs (2), nephritis (10); placentitis with abortions (7), interstitial focal lymphocytic nephritis (11), abscesses in various organs (7) and inflammations in various organs, especially lymphadenitis and mastitis (10). In 19 sheep parasitic diseases, especially dicrocoeliosis (9), fasciolosis (9), and moniesiosis (6) were noted.

In 100 rams experimentally infected with brucellosis species, granulomas were found in the liver (40) of giant cells (10), in lungs (60) with giant cells (30), in testes (14) with giant cells (2), and seminal vesicle (28) with giant cells (4). In reproductive organs, periorchides (66), orchides (36), and epididymides (52) were found. Also frequent was the occurrence of various parasites, as in the group of sheep.

In 28 young rams free of parasitoses except for an isolated occurrence of moniesiosis experimentally introduced by brucellosis species PZ 1267, PZ 737 (B-17), TM 232-4, and PZ 1276, which were isolated by Niznansky from cases of infectious epididymitis of rams, there were produced also granulomas in the lungs, liver with isolated occurrence of giant cells of the Langhans type, periorchides, orchides, and epididymides, and also interstitial focal lymphocytic nephritis and abscesses in various organs.

Granulomatose infections in the lungs, liver, spleen, kidneys and testes were proved both in rabbits diseased spontaneously with a cultivation occurrence of Brucella suis and in rabbits which were experimentally infected (intraperitoneally) with species Brucella suis, Brucella bovis and sporadically (mainly in the spleen) in young rabbits born to females which had been experimentally infected with species B. suis before impregnation. The females did not abort, but gave birth to live rabbits. Besides ganglionic infections, orchides, metridies, and abscesses in the lungs, under the skin, in the liver and lacteal gland were found.

In guinea pigs infected experimentally with brucellosis species H₁₃, PZ 737, TM 232-4, TM 38/56, PZ 1276, and 1267, the formation of granulomas was most prominent in the liver, less so in the lungs, spleen, and kidney. Sexual abnormalities in the form of periorchides, epididymides, and orchides were found in a group of guinea pigs experimentally infected with the species Brucella suis, periorchides and epididymides in group PZ 737, and periorchitis only in a guinea pig infected with the species Brucella bovis. A frequent phenomenon in all groups was focal desquamative catarrhal bronchopneumonia with infrequent abscesses in various organs. Granulomatose infections were found in all groups of guinea pigs experimentally infected with the species Brucella suis and Brucella bovis for comparative reasons.

In rats experimentally infected with the PZ-1267 species, suppurative processes in various organs were predominant in three cases, whereas the formation of granulomas was noted only in the lungs in two cases. Periorchides were found in two rats. Rats experimentally infected with the brucellosis species TM 38/56 showed a distinct granulomatose reaction in the kidneys (4) and lungs (2). In the reproductive organs, periorchides (3) and epididymides (3) were found. In rats of the group which was

Table 2 (Left side)

A comprehensive table of granulomas found in the individual kinds of animals that were diseased spontaneously and infected experimentally by brucellosis species

	Sheep		Rams					Rabbits						
	Various brucellosis species	PZ-1267	PZ-737	TM-232-4	PZ-1276	Various brucellosis species	Spontaneous disease; agglutination to BAB m antigen positive	Brucella bovis	Brucella suis	From mother experimentally infected with species B. suis	Spontaneously diseased with B. suis	B-19	B. bovis	B. suis
Liver	19a	10	10	10	10	100	2	5	6	1	2	4		10
	16e	4	5	5	5	40								
Lungs	12a	20	20		10	300								
	2c													
	1e, 6d	5	2	3	3	600	1	3	4		3	2		6
Spleen								3	4	5	2	3		
Kidneys						20		2			1	1		
Testes						14								
						40								
Seminal vesicle						28								7
Uterus														
Other organs														
Periorchitis		4	6	6	5	66	3						1	2
Orchitis		3	2	3	4	36	2	1	7	1	2			4
Epididymitis		3	3	4	2	52	2							5

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GRANULOMAS

Guinea pigs						Rats		Mice		Notes	Explanations
	PZ - 737	TM - 232 - 4	TM 38/56	PZ 1276	PZ 1276	PZ 1276	TM 38/56	B. bovis	B. bovis		
H - 13											
4	9	5	4	5	5		TM 38/56	3	7		Langhans giant cells found in granulomas
1			2	1		2			1		a- lymphoid granuloma b- lymphoido-epitheloid granuloma
	2	1	2	2				2			c-epitheloid granuloma d-giant cell granuloma
2	2			1	1	4					e-eosinophil granuloma f-fibrotized granuloma
									1		
									1		
	1					2	3	1			
	2					3		1			

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infected with the species *Brucella bovis*, granulomas appeared in the liver (3) and spleen (2); whereas in the reproductive organs periorchitis (1), epididymitis (1), and (in various organs, especially in lungs and cerebral ganglia (2), inflammations were observed.

Granulomatose reactions of the microgranuloma type which, unlike other kinds of investigated animals, resulted in death were prominent in the liver (7), less so in the lungs (1) and seminal vesicles (1) of white mice.

More details on the examined animals concerning the occurrence of granulomas will be found in Table 2. The microscopic structure is given in pictures 1-10.

Discussion

The results obtained demonstrate that both in sheep and in laboratory animals, brucellosis granulomas are observed. These, after having been differentiated from eosinophil parasitic granulomas and other specific granular inflammations, may serve as a valuable instrument in the macro- and microscopic diagnosis of brucellosis of sheep and laboratory animals. They may be used in the laboratory diagnosis of brucellosis in the evaluation of a biological experiment as well as in the differential diagnosis of tuberculosis, which is often accompanied by granulomas in the RES organs, especially in spleen, liver, and lungs (quoted by Willis, H. S. and Cummings, M. M., and others.

The occurrence of granulomas in sheep, as far as the structure and the location are concerned, agree with the specifications of Juskovec and Cernjak. The occurrence of giant cells in cerebral ganglia, mentioned by Juskovec, was not noted in sheep.

In rabbits a variability of granulomas with respect to location and structure was observed. The granulomas found showed a structure described by Bendtsen, Christiansen and Thomsen, Roux and Bouvier, and Tudoriu. There were also found granulomas of the kind noted by Tudoriu, Albertini and Lieberher, and Lofler and Albertini. Very characteristic was eosinophilia and pseudoeosinophilia, as mentioned by Roulet.

Polymorphism of the granuloma structure, as described by Aiello, was noted. Ganglionic infections of the liver and RES organs of rabbits, however, should be differentiated from tuberculosis, pseudotuberculosis, chronic abscesses, staphylococcosis, tularemia, and (in liver especially) from coccidiosis and cysticercosis. The similarity of the pathomorphological variations of pseudotuberculosis, staphylococcosis and brucellosis was pointed out by Bendtsen, Christiansen and Thomsen, and Peti. According to them, the suppurative infections of the testes and seminal vesicles are most likely symptomatic of brucellosis, although suppurative infections were noted in the testes even by pseudotuberculosis (relatively less frequent).

Most frequent in all groups of guinea pigs was the occurrence of granulomas in their liver; these changes were observed in 61% of the investigated cases. Even though granulomas may develop in guinea pig liver

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also by tuberculosis pseudotuberculosis, and salmonellosis, the mentioned granulomas may be ascribed considerable significance after differentiation from ganglia of different etiology. The structure of the granulomas was identical with that described by Braudé, Moulton and Meyer, Parnas, and Versilov and Kokorin. In other organs the occurrence of the mentioned granulomas was irregular but relatively frequent, and showed that the morphological symptomocomplex of brucellosis in guinea pigs has a relatively frequent granulomatose character. A very valuable differential diagnostic aid in the differentiation of the mentioned granulomas which can occur in the liver, is the changes in the reproductive organs which, according to our results, may be of decisive importance for the differentiation of the mentioned diseases from brucellosis. Tubercular, and pseudotubercular changes in the reproductive organs are, in comparison with brucellosis, very rare, although they may also occur.

Comparing the occurrence of granulomas or microgranulomas of liver, it was found that they were more frequent in mice than in rats and were already observed during the first days after experimental infection, as stated by Roulet.

The granulomatose morphological symptomocomplex in the RES organs with concurrent changes in the reproductive system by brucellosis in both sheep and laboratory animals, may serve as a very valuable diagnostic aid in the differentiation of brucellic and parasitic liver granulomas.

As far as the structure of the brucellic granulomas is concerned (taking into consideration the cell polymorphism which was observed by a large majority of the authors and was also confirmed by our investigation), we assume that this can be dependent on the length of the process, as described by Janovskij in the metamorphosis of the lymphoido-reticular mesenchymal cells. In addition to the time factor and the kind, quantity, and virulency of the germs, different specific and individual resistances of the animals as well as the environment, which can affect the diseased macroorganism and the pathogenic microorganism (as stated by Cernjak), may play an important part. Granulomas were found predominantly in animals which did not die of themselves, whereas in those which perished, non-specific inflammatory process were prevalent. This is worthy of attention from the point of view of immunology.

As far as the frequency of the granulomatose infections is concerned we can not agree with the opinion of Smith and Jones that the occurrence of granulomas by brucellosis is sporadic, since our investigation has proved that these formations appear in a considerably greater number than recorded in the bibliography.

Summary

For the purposes of the study of morpho- and histogenesis of brucellic granulomas in certain organs, 136 sheep and 168 various laboratory animals were examined.

Granulomas were found most frequently in the liver, lungs, spleen, kidneys, and peritoneum, but also were found in the reproductive organs, testes, seminal vesicles, and uterus both in sheep and laboratory animals.

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The most stable part of a brucellic granuloma is the epithelioid cells, giant cells of various types, and lymphoid cells surrounded in fine reticular stroma. Apart from these, the occurrence of capillaries, eosinophil granulocytes, neutrophil polymorphonuclear leucocytes, and reticular cells is also very frequent. The periphery is enclosed in a zone of a non-specific granular tissue in which plasmatic cells, monocytes, vessels, fibroblasts, and fibrocytes may be visible. The center frequently necrotizes; in this the granuloma resembles an abscess.

In the development of brucellosis granulomas, perivascular cellular infiltrates in the RES organs are formed. In latter stages lymphoid, lymphoido-epithelioid, and (seldom) giant cellular granulomas which contain a great quantity of eosinophil granulocytes and vessels are formed. The center of the mentioned granulomas shows various degrees of necrobiosis up to necrosis. Frequently both the peripheries and the center show symptoms of a total or partial fibrosis.

In guinea pigs experimentally infected with brucellosis species PZ 1276, TM 38/56, H-13, TM -232-4, and PZ 737 (B 17), fibrosis was prominent after 48-150 days. In rabbits experimentally infected with species B. abortus and B. suis, fibrosis was observed as late as 390 days after the infection.

A brucellosis granuloma from the morpho- and histogenetic point of view represents a product of the RES reaction originating by a metamorphosis of reticuloendothelia with a consequent transformation into highly differentiated types of cells.

The occurrence of granulomas in the RES organs and the concurrent appearance of inflammatory modifications suggest brucellosis, with a high degree of probability.

In sheep the occurrence of brucellosis granulomas in the RES organs is very frequent. In liver, however, these are in practice confused with and taken for parasitic eosinophil granulomas; a differentiation should be worked out.

Captions to Photographs

Photograph 1 shows: Peribronchial lymphoido-epithelioid granuloma of lungs with a necrotic center. Rabbit L-1/9/58, exposed to infection by .6 ml. of standard solution of species B. Abortus; killed after 270 days. Bacteriological result negative. Magnification 10 x 12. HE dye.

Photograph 2 shows: Peribronchial granuloma. Young ram 029, L-1/423/58, exposed to infection with species PZ 1267. Killed 30 days after infection. Agl. 1/10240++, RVK 1/L+++. Bacteriological result negative. Magnification 10 x 12. HE dye.

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Photograph 3 shows: A part of a periportal granulomas conglomerate. Young ram 029, L-1/423/58, infected by species PZ 1267; killed 90 days after infection. Agl. 1/10240q++, RVK 1/1++++. Bacteriological result negative; magnif. 10 x 12; HE dyd.

Photograph 4 shows: Liver granuloma with a thin layer of fibroblasts on the periphery. In the upper and lower left corners microgranulomas are visible. Young ram 016 L-1/413/58 infected by species TM 232-4, killed 15 days after infection. Interstitial epididymis and periorchitis. Parasites in organism have not been found. Agl. 1/10240++, RVK: 1/1++++. Magnif. 10 x 12; HE dye.

Photograph 5 shows: Lymphocytic non-suppurative epididymis with obliteration of seminal vesicle ducts (to the right). Ram L-1/113/56 contaminated by infective epididymis species. Granulomas in kidneys, lungs, liver. Bacteriological result negative. Magnif. 10 x 12, HE dye.

Photograph 6 shows: Interstitial lymphocytic nephritis. Ram exposed to 5 ml of intraperitoneal emulsion of standard 38/57 species. Interstitial lymphocytic epididymis and fibrous periorchitis.

Photograph 7 shows: Liver granulomas conglomerate with a necrotic center. Rabbit L-1/224/58; exposed to infection by .6 ml emulsion of standard solution of B. suis; killed after 210 days. Magnif. 10 x 12. HE dye.

Photograph 8 shows: Subcapsular epithelial spleen granuloma. Guinea pig No L-1/374/58, infected intraperitoneally by species B. suis; killed 15 days after infection. Magnif. 10 x 12, HE dye.

Photograph 9 shows: Submucous lymphoido-epitheloid vagina granuloma. Rabbit L-1/4/58, infected by intaperitoneal emulsion of standard B. suis solution. Killed after 390 days. Bacteriological result negative. Magnif. 10 x 12; HE dye.

Photograph 10 shows: Liver granuloma disintegrated in the center (to the right). Young ram 014, L-1/304/58, infected by species B. suis. Killed 15 days after infection. Magnif. 10 x 12, Granulomas of a similar structure found in lungs and kidneys. No parasites in organism discovered. Agl. 1/2560++, RVK 1/1++++. Bacteriological result negative. Magnif. 10 X 12; HE dye.

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THE EXAMINATION OF BLOOD SERA FOR BRUCELLOSIS BY
THE RING REACTION

Following is the translation of an article by Docent Jaroslav Drazan, DVM, and Vojtech Madr, DVM, in Veterinarni medicina (Veterinary Medicine), No 12, Prague, December 1960, pages 883-891.

Introduction

The development of mass technology in stockraising calls for an introduction of those diagnostic methods which would make it possible to examine the whole stock, or possibly all sensitive animals of a certain area, within a short time. This requires the application of diagnostic methods which enable us to carry out consistent prevention of especially those contagious diseases which may endanger primarily large scale stock raising. Among these belong tuberculosis, brucellosis, and inflammations of lacteal glands. The successful prevention of these infections depends on a fast, reliable, and practical diagnosis with the help of which the contagion situation in whole districts may be easily controlled.

The examination for brucellosis is practiced at present by testing blood serum by means of a slow agglutination reaction or the addition of complement; in areas free from brucellosis, also by a ring reaction in the milk of milk cows.

It appears, however, that after the socialization of our agriculture has been completed and modern mass technology introduced into most types of production, it will probably be rather difficult to provide for a regular examination of all animals by the present classical serological methods.

It is therefore a pressing task to work out such methods which would make it possible reliably to control the health condition of animals with relation to brucellosis. The demand for a rapid diagnosis could be fulfilled by allergenodiagnosis, a fast agglutination reaction, or the ring reaction, which we dealt with in our preceding works. Studying the principals of the ring reaction, we arrived at the conclusion that with the help of the ring reaction (RR), antitoxins may be proven not only in milk but also in blood serum.

The possibility of examining blood sera by means of RR was mentioned by Canic as early as 1939. Later, in 1952, Bendtsen published the results of an examination of the sera of animals afflicted by brucellosis by means of RR. In this examination he used his original antigen. Nyiredy compared applying Benedek's method, the results of sera examination by means of RR

with the classical slow agglutination in 22,000 animals of various kinds. By this comparison, identical results of the RR and the slow agglutination reaction were obtained. The author considers RR more sensitive and exact. Benedek, in his work in 1954, used in the RR examination antigens of various types of Brucellae dyed with 2,3,5-triphenyltetrasoliumchloride. He notes that by means of this examination method a typification of Brucellae was made possible. Also, Daigeler, Brommel, and Wiest examined the sera of animals by means of the RR and compared the results with several serological reactions.

Independently, on the works of the above-mentioned authors we developed in the course of 1955 a method of the examination of blood sera for BAB by means of RR.

One of the goals of our study of the serological reactions in brucellosis was clarification of the principles of the origin of RR. Fleischhauer, Orlov, and Morjakova, Benedek, Bendtsen, Krejci, and a number of others explain the origin of the RR by a conglomeration of the dyed bacteria in positive milk through the action of the antitoxins into agglutinates, which are carried by the rising fat globules to the surface and thus form a colored layer of cream.

Comparing the results of the slow agglutination using the whey of one sample of commercial milk with the results of the RR, it was striking that by RR BAB antitoxins were proven in this milk very easily, whereas by slow agglutination we were unable to ascertain the agglutination titer even in a 1:5 solution. The sample was taken from the total contingent of milk from one farm in which one cow was brucellotic and 70 cows negative. Milk of the positive cow was in the total contingent diluted by negative milk in a ratio of 1:28. This result lead to the conclusion that a major part of the specific antitoxins was confined to cream, and possibly a small part only was dispersed in milk. This presumption of ours was confirmed by an experiment in which cream was carefully removed and substituted by cream from the negative milk, after which the RR was repeated. This time, the RR was dubious. This conclusion was also confirmed by an experimental, gradual saturation of antitoxins from the positive milk by negative cream. (Positive milk was washed by negative cream; the cream was then removed and the skimmed milk again washed by negative cream. This procedure was repeated four to six times, and the result was that the agglutinins were all but removed from the milk.)

By the repeated washing of cream obtained from positive milk by a physiological solution, it is possible, on the other hand, to remove from such globules the agglutinins so that such cream, when mixed with negative milk, shows a negative RR.

Finally, by the further study of the RR in positive cream diluted by physiological solution in a ratio of 1:5, we proved that the appearance of positive reactions in these samples had no relation to the presence of agglutinins in the physiological solution, and that there was direct adsorption (agglutination) of brucellae to the surface of the globules whose albuminous cover contains specific antitoxins. These

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specific antitoxins have to the albuminous cover of such globules a definite special affinity, so that evidently a major part of the free agglutinins contained in the positive milk is adsorbed by the fat globules of the negative milk when the positive milk is mixed with the negative. All the cream from such mixed milk has then a character of cream from brucellotic milk.

The experience that the agglutinins in milk are tied to fat globules, or that by fat globules the agglutinins can be adsorbed from milk and that they even can be removed from such globules was also confirmed by experimental addition of positive brucellotic blood serum into negative milk and the subjection of such milk to the RR. A positive RR was obtained when the added serum contained specific agglutinins. The intensity of the RR depended on the quantity of agglutinins in the added serum (i.e., concentration of the agglutination titer). The RR in milk occurred not only after the addition of positive serum but after the addition of any liquid with a content of agglutinins to the negative milk (sperm, or other secretions of brucellotic animals).

After gaining this fundamental knowledge about the origin of the RR, we arrived at the conclusion that the agglutinins are adsorbed in milk on the protein covers of fat globules on one hand, or remain free (not tied to the fat globules) in milk, on the other hand. The agglutinins adsorbed to the surface of such globules directly tie the colored brucellae, whereas the free agglutinins conglomerate the dyed antigen into agglutinates which are partially carried away by the fat globules into the cream, but mostly sedimentate and are found at the bottom of the test tube as colored agglutination. This fact leads us to the development of a modified RR by means of which specific brucellotic antitoxins are proved in the examined serum.

Serum examination method

To ml [milliliter] of previously-tested negative milk, .2, .15, .1, and .005 ml of serum that is to be tested is added in a test-tube. The contents of the test-tube should be agitated and placed for 10-15 minutes into a thermophore. During this time the test-tubes should be mildly agitated several times so that a rapid adsorption of agglutinins to the fat globules can be obtained. After that, 1 drop (approximately .05 ml) of color BAB antigen should be added to the test-tubes, the contents again agitated, and the test-tubes placed in a thermophore. After a one-hour incubation the reaction is evaluated.

Components used in RR serum examination are:

- I) Negative milk (fresh, raw, cow's milk);
- II) Color BAB test,
- III) Examination serum.

I) Milk used for the examination of sera should be obtained only from healthy cows which have not been infected by brucellosis; samples for examination should be taken beforehand.

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Milk for the examination of sera by RR is suitable only on the condition that:

- 1) The fat content is approximately 3-4%,
- 2) The Fleischhauer RR test is absolutely negative,
- 3) A negative reaction is obtained even after the addition of various quantities of a serum which is with regard to brucellosis quite negative and which was tested by serological reactions beforehand,
- 4) The RR is positive after the addition of positive BAB serum whose agglutination titer has a minimum value 320.

The process of the milk examination

<u>Quantity of the examined milk</u>	<u>Quantity of the Serum</u>	<u>Color test</u>	<u>Result of the reaction</u>
1. 1 ml	-	.05 ml	negative
2. 1 ml	.1 ml neg.	.05 ml.	negative
1 ml	.2 ml neg.	.05 ml	negative
1 ml	.4 ml neg.	.05 ml.	negative
3. 1 ml	.05 ml pos.	.05 ml	pos.++up to +++
1 ml	.10 ml pos.	.05 ml	pos. +++
1 ml	.20 ml pos.	.05 ml	pos. +++

In this manner we searched for suitable milk from cows from which necessary quantities of milk for serum examinations are obtained. Milk should be kept in a clean, sterile container in order to prevent its contamination. Milk which is suitable for serum examination can be kept (preserved) in a refrigerator in large quantities for a period at least one month. As the most suitable preservative, we used Formalin in the final concentration of .25 - .5% in milk.

II) Color BAB test. The suitability of the test which will be used in the RR serum examination is to be tested beforehand by absolutely negative milk so that the specificity of the antigen can be determined. For the RR serum examination, a test older than three months is not to be used. In examining the sera, we used hematoxylin antigen manufactured in Bioveta at Ivanovice na Hane, according to Hermann, and an antigen of our own production dyed by 2,3,5-triphenyltetrasoliumchloride, according to Bendtsen.

In comparative tests, we did not find any substantial difference of results in the application of these two antigens. The distinctness and the results of the reactions, especially in comparison to the agglutination reaction, are considerably dependent on the quality and quantity of the antigen used. (In measuring of the antigen (dropping) it is necessary to use always the same unimpaired pipets.) In 1 ml of

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test, a constant number of brucellae must be present. The cultures of brucellae used in the preparation of the antigen must be absolutely pure and must not contain R-forms.

III) Examined serum. Each serum is to be examined in four concentrations. To 1 ml of milk in the first test tube, .05 ml; in the second, .1 ml; in the third, .15 ml; and in the fourth, .2 ml of serum is added. By this, approximately the following concentrations will be obtained: 1:20, 1:10, 1:7.5, and 1:5.

Evaluation of the reactions: Three "+" signs are used for reactions in which a 2-3 mm layer of colored cream is formed on the surface of the milk. The milk must retain its natural color. Two "+" signs are used for reactions in which a colored cream layer is formed, as in the preceding reaction, but the milk is not completely discolored. These two reactions are considered positive. One "+" sign is used for dubious reactions in which an unsatisfactorily colored cream layer is formed and the milk is practically not discolored at all. The "+" sign is used for reactions in which both the milk and the cream are equally colored; on the bottom of the test tube sometimes a small agglutination is formed. In negative reactions the antigen remains dispersed in the milk, and the cream at the surface remains white. Both of these two latter reactions are considered negative.

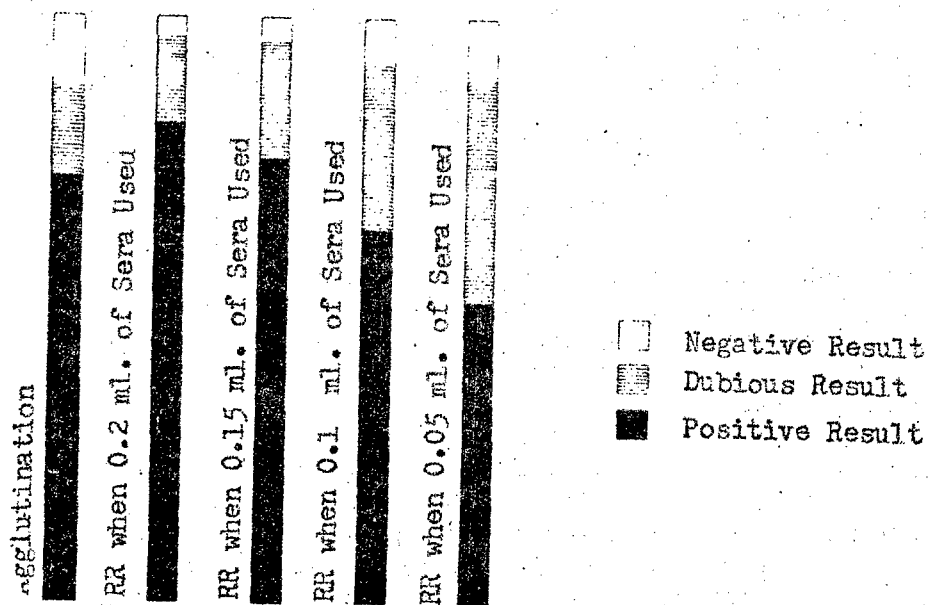
In dubious and negative results of the RR, we take into consideration the possible agglutination at the bottom of the test-tube; the result of the RR is again evaluated after 12 or 24 hours.

Control: the results of the RR were compared with the results of the slow agglutination reaction which was carried out in the following parallel manner. The basic concentration of the serum examined by physiological solution was prepared in a ratio of 1:5 (.2 ml of serum + .8 of physiological solution). In the following test tubes, the concentration was gradually increased up to 1:2560. By the addition of an equal volume (.5 ml) of 2.5% bacterial test, an initial concentration of 1:10 and a final concentration of 1:5120 was obtained. The final concentration of the bacterial suspension in each test-tube was 1.25%. In agglutination, we used a test of the Ivanovice Bioveta production. The stands with the test tubes were placed for 24 hours into a thermophore and after another 24 hours in room temperature, the reaction was evaluated. Positive was considered a complete agglutination of bacteria in a concentration of serum 1:80 (+++) and dubious agglutination, in a concentration of 1:40 (+++). A concentration of 1:20 and less was considered a negative reaction.

In the above manner, we examined and compared with the results of agglutination the reactions 5,792 sera of cattle, sheep, and horses. Out of this number, 2,815 samples of sera were taken from the branch of the SVVU (Statni Vyzkumny Veterinarni Ustav -- State Veterinary Research Institute) at Brno.

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examination, .2, .15, and .05 ml. quantities of serum, added to 1 ml of raw milk from healthy cows, were used. A color antigen according to Hermann or Bendtseh was used in this reaction. The reaction was evaluated after one hour. By using .15 ml of serum and a one-hour incubation in a thermophore, the results of the examination by the RR are essentially identical with those of the Wright slow agglutination reaction.



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Results

The results obtained from the examination of 5,792 sera of cattle, sheep, hogs, and horses by the RR show that most suitable results of the RR are received, in comparison with the slow agglutination reaction, when .15 ml of serum is tested. The quality test of the serum mentioned gives a reaction by which the examined serum can be adequately evaluated. Such an evaluation may prove satisfactory in a fast orientation examination. For the establishment of the degree of the agglutination titer which contributes to the evaluation of the infection process, it is, however, necessary to examine serum in various concentrations.

In the quantitative evaluation of the agglutinins in the serum, the evaluation of the intensity of the RR is another very helpful factor. The reaction marked with three "+" signs in case .2 ml of serum is used corresponds very often to the agglutination titer 40, sometimes even 80, and then to all higher titers. The weaker RR (++) corresponds to a lower agglutination titer (the quantity being the same as in the previous case), 20-40. A RR marked by one "+" sign indicates the titer found in the serum in a concentration of 1:10 to 1:20. A RR marked with three "+" signs when .15 ml of serum is used corresponds to an agglutination titer of 80, and sometimes even to a titer of 40. A RR marked with three "+" signs when .1 ml of serum is used corresponds to the 80 agglutination titer, sometimes to the 160 titer, and, naturally, to all higher titers. A RR marked with three "+" signs when .05 ml of serum is used is examined in sera which show in the slow agglutination reaction a titer of 160 to 320.

The results and intensity of the ring reactions at a constant quantity of examined serum correspond to a wider range of respective titers than we have mentioned. This range is to be accounted for by the varying quality of milk used in the RR, the quality of antigen, and, last but not least, by errors made by the tester in the course of the reaction. Even biochemical peculiarities of the tested sera may sometimes be of importance here.

An experienced worker who evaluates the final results of the reactions, not only by the concentration of the sera but also by the intensity of the reactions, can determine the approximate quantity of antitoxins in the blood serum. By means of the described method, it is possible to obtain within two hours results which are often more convincing than those of the slow agglutination reaction. The simplicity of application and evaluation of this reaction is certainly a great advantage.

Summary

- 1) This work describes the principle of the Ring Reaction and suggests a method of blood serum examination by the RR.
- 2) A modification of the RR was used in 1954-55 in examining 5,792 samples of blood sera of cattle, horses, hogs, and sheep. In the

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I. Result of RR when 0.2 ml. in 1 ml. of milk was used
 I. Result of RR when 0.2 ml. in 1 ml. of milk was used

Result of		Agglutination Titer									
Result	Reaction	0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	and over
Ring Reaction		136	206	384	874	1132	936	1146	251	727	and over
+++	3709			23	529	698	878	1146	251	727	
++	1023		139	323	532	392	58				
+	857	112	139	361	293	64					
±	179	112	67								
	24	24									
Given in Percent											
Result of		Agglutination Titer									
Result	Reaction	0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	and over
Ring Reaction		2,35	3,56	6,63	15,08	19,54	16,16	19,78	4,35	12,55	and over
+++	58,61			0,28	5,59	16,73	14,63	19,78	4,35	12,55	
++	18,03		2,33	0,28	5,39	10,63	1,53				
+	19,19	0,81	2,33	5,16	9,49	2,21					
±	2,74	0,52	0,39	1,19							
	2,03	1,54	0,49								

II. Result of RR when 0.15 ml. in 1 ml. of milk was used
 II. Result of RR when 0.15 ml. in 1 ml. of milk was used

Result of		Agglutination Titer									
Result	Reaction	0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	and over
Ring Reaction		136	206	384	874	1132	936	1146	251	727	and over
+++	3709										
++	1044		135	209	330	428	88				
+	1112	47	135	269	550	128					
±	159	49	43	69							
	117	89	28								
Given in Percent											
Result of		Agglutination Titer									
Result	Reaction	0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	and over
Ring Reaction		2,35	3,56	6,63	15,08	19,54	16,16	19,78	4,35	12,55	and over
+++	58,61			0,28	5,59	16,73	14,63	19,78	4,35	12,55	
++	18,03		2,33	0,28	5,39	10,63	1,53				
+	19,19	0,81	2,33	5,16	9,49	2,21					
±	2,74	0,52	0,39	1,19							
	2,03	1,54	0,49								

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III. Result of the RR when 0.1 ml. in 1 ml. of milk was used

Result of Ring Reaction		Agglutination Titer									
		0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	and over
		136	206	384	874	1132	936	1146	251	727	
+++	2435					97	357	1003	251	727	
++	1272				192	384	553	143			
+	1617		60	198	682	651	26				
±	337	27	124	186							
-	131	109	22								
Given in Percent											
Result of Ring Reaction		Agglutination Titer									
		0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	and over
		2,35	3,56	6,63	15,08	19,54	16,16	19,78	4,35	12,55	
+++	42,05					1,68	6,16	17,31	4,35	12,55	
++	21,95				3,31	6,62	9,55	2,47			
+	27,92		1,04	3,42	11,77	11,24	0,45				
±	5,82	0,47	2,14	3,21							
-	2,26	1,88	0,38								

IV. Result of the RR when 0.05 ml. in 1 ml. of milk was used

Result of Ring Reaction		Agglutination Titer									
		0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	and over
		136	206	364	874	1132	936	1146	251	727	
+++	1696					61	202	558	148	727	
++	1260				17	222	443	475	103		
+	2225			188	784	849	291	113			
±	419	21	129	196	73						
-	192	115	77								
Given in Percent											
Result of Ring Reaction		Agglutination Titer									
		0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	and over
		2,35	3,56	6,63	15,08	19,54	16,16	19,78	4,35	12,55	
+++	29,28					1,05	3,49	9,63	2,56	12,55	
++	21,75				0,29	3,83	7,66	8,19	1,79		
+	38,41			3,24	15,53	14,66	5,02	1,96			
±	7,25	0,37	2,23	3,39	1,26						
-	3,31	1,98	1,33								

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V. Final Results of Slow Agglutination and Ring Reaction Examination of Sera

Result of the Reaction	Agglutination	RR when Quantities of Sera used were			
		0,2 ml	0,15 ml	0,1 ml	0,05 ml
Positive	4192	4732	4404	3707	2956
Dubious	874	857	1112	1617	2225
Negative	726	203	276	468	611

Result of the Reaction	Agglutination	Application of Sera in RR, in %			
		0,2 ml	0,15 ml	0,1 ml	0,05 ml
Positive	72,38	81,7	76,04	64,0	51,03
Dubious	15,08	14,78	19,19	27,92	38,41
Negative	12,54	3,52	4,77	8,08	10,56

VI. Relation between the Agglutination Titer and the RR Intensity in Various Concentrations of Sera.

Agglutination Titer	Average Result of RR when Quantities of Sera used were			
	0,2 ml	0,15 ml	0,1 ml	0,05 ml
0	—	—	—	—
1 : 10	+	+	+	+
1 : 20	+	+	+	+
1 : 40	++	++	+	+
1 : 80	+++	++	++	+
1 : 160	+++	+++	+++	+
1 : 320	+++	+++	+++	+++
1 : 640	+++	+++	+++	+++
1 : 1280	+++	+++	+++	+++

10,132

-- END --